Akirin1, a nuclear factor, induces cardiac hypertrophy in mice via SRF and miR-1

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ABSTRACT

Akirin1 is a small ubiquitously expressed protein, which belongs to a family of highly conserved nuclear proteins called akirins. Due to its nuclear localization and propensity to interact with other proteins, Akirin1 is speculated to regulate transcription of its target genes as a cofactor. Previous studies have shown that Akirin1 is a downstream target of myostatin, which is a negative regulator of myogenesis. Akirin1 was expressed at higher levels in mice lacking myostatin and consistent over-expression of Akirin1 induced hypertrophy in C2C12 myotubes in vitro. While Akirin1 has been mainly studied in skeletal muscle, its role in other muscle tissues remain largely unknown. Here, we studied the function of Akirin1 specifically in cardiac muscle using Akirin1 knock-out mice as a model system. The results of this study show a novel function of Akirin1 in cardiac muscle. Through histological analysis, we show that Akirin1 knock-out mice display larger ventricular cavities in the heart as usually seen in left ventricular hypertrophy (LVH). Compared to wild-type, Akirin1 knock-out cardiac muscle showed post-transcriptional up-regulation of serum response factor (SRF), a critical molecule known to maintain the cardiac muscle homeostasis. Consistently, Akirin1 knock-out cardiac muscle displayed transcriptional down-regulation of miR-1 and thus up-regulation of its target genes i.e. β-MyHC and ANP. Finally, ablation of Akirin1 also resulted in the activation of signaling molecules involved in IGF-1/Akt/mTOR pathway that is implicated in increased protein synthesis, all suggestive of cardiac hypertrophy phenotype. Overall, we propose a novel role of Akirin1 in cardiac muscle homeostasis via regulation of SRF and miR-1 expression.

KEYWORDS: Akirin1, cardiac hypertrophy, cardiac muscle, sarcomeric proteins

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INTRODUCTION

Cardiac muscle has the tendency to undergo structural changes in response to stress factors like hemodynamic load and/or cardiac injury. The cardiac muscle undergoes a series of structural changes, which eventually alter the dimension and function of the heart. Such changes undertaken by the cardiac muscle is constitutively termed as cardiac remodeling. Depending on the type of cardiac remodeling, the cardiac muscle can either increase in mass (hypertrophy) or decrease in mass (atrophy). The adult human heart is typified on its growth characteristics based on the functional load and stimuli it receives. In order to counteract the changes in the functional overload, the cardiac muscle elicits a hypertrophic response. Cardiac hypertrophy can be broadly classified into two types based on the type of hypertrophic stimulus-physiological hypertrophy and pathological hypertrophy (Cohn et al., 2000). Physiological hypertrophy is a beneficial hypertrophy that occurs in response to physiological stimuli like regular physical activity or heavy exercise training or during pregnancy (Mone et al., 1996). When there is a hemodynamic load on the heart walls due to chronic physical exercise, the cardiomyocytes are stretched which leads to synthesis of new contractile proteins and usually the new sarcomeres are arranged in parallel. This increases the muscle thickness of the heart wall, thereby increasing the contractile ability and eventually the cardiac output (Wakatsuki et al., 2004). Thus, physiological hypertrophy is a compensated growth to normalize the wall stress and usually reverts back to normal on cessation of exercise regime (Zak, 1984), with either normal or enhanced cardiac output. Physiological hypertrophy can be further subdivided into two categories depending on the type of hemodynamic overload, the eccentric and concentric hypertrophy. The eccentric physiological hypertrophy occurs in case of aerobic exercises like running and swimming due to volume load on the heart walls, leading to the addition of new sarcomeres next to each other in the cardiomyocytes and dilating the heart walls. On the contrary, concentric hypertrophy is prevalent during static exercise like weight lifting due to pressure load on heart wall, resulting in addition of sarcomere in parallel to each other, thereby increasing the thickness of the heart walls (Pluim et al., 2000) Zak, 1984).

Physiological hypertrophy is mainly induced by IGF1 (Insulin Growth Factor-1) -phosphoinositide-3 kinase (PI3-K) pathway. IGF-1 growth factor released in response to exercise training interacts with its receptor IGF1R. This further activates the PI3-K leading to activation of protein synthesis pathway resulting in cardiac cell growth (Toker and Cantley, 1997). Mice having increased cardiac IGF-1/PI3-K have normal life span but develop physiological hypertrophy with increased cardiac output (Reiss et al., 1996; Shioi et al., 2000). On the contrary, mice with reduced IGF-1/PI3-K signaling show reduced heart size with a dampened hypertrophic response to swim training (Luo et al., 2005; Shioi et al., 2000). These studies indicate that IGF-1/PI3-K pathway is pivotal in modulating physiological hypertrophy induced by exercise training (Luo et al., 2005; Shioi et al., 2000). Although the IGF1/PI3-K pathway seems to play a defining function in inducing physiological hypertrophy, it might also be activated in pathological conditions as well. A different kind of cardiac remodeling occurs in response to hemodynamic overload due to cardiomyopathies and chronic heart diseases like aortic stenosis, hypertension, valve diseases and myocardial infarction known as pathological hypertrophy (Zak, 1984). Pathological hypertrophy is characterized by increase in the heart muscle wall thickness due to the hemodynamic stress along with pathological stimuli such as production of collagen by cardiac fibroblasts, inflammation and apoptosis/necrosis of cardiomyocytes (Volders et al., 1993). All these factors affect the contractility of cardiac muscle and eventually the cardiac output. These changes are irreversible and eventually lead to heart failure and increased mortality (Mejls et al., 2010; Olivetti et al., 1997). Re-expression of fetal genes likes
**Atrial natriuretic peptide (ANP), β-MyHC and skeletal alpha-actin** is usually seen during pathological hypertrophy and not during physiological hypertrophy. In pathological hypertrophy, the compensatory growth is eventually decompensated leading to dilated cardiomyopathy and heart failure (Fagard, 1997; Pluim et al., 2000). A type of pathological hypertrophy categorized as concentric hypertrophy is mainly caused due to a pressure overload, for instance, hypertension or aortic stenosis leading to thicker heart walls and relatively small cavities eventually increasing the systolic wall stress (Grossman et al., 1975). Oppositely, an increase in diastolic wall stress results in eccentric hypertrophy due to stimulus leading up to volume overload, as seen in aortic regurgitation, thus dilating the heart cavities and comparatively thinner walls (Grossman et al., 1975; Pluim et al., 2000).

However, there is a possibility of cross talk between these two main signaling pathways in developing either type of cardiac hypertrophy. The precise signaling pathway and cardiac remodeling events differentiating physiological from pathological cardiac hypertrophy still remain a clinical challenge in cardiovascular research. Recent studies in the field have shown that myostatin, a member of transforming growth factor β-superfamily is expressed in heart predominantly in the purkinje fibers and cardiomyocytes (Sharma et al., 1999) and previous studies highlight a role of myostatin in inhibiting IGF-1 induced physiological hypertrophy in the heart, thus acting as a chalone for heart organ development (Shyu et al., 2005). On the contrary, Rodgers et al. showed that myostatin null mice exhibited physiological hypertrophy and loss of myostatin induced eccentric hypertrophy with enhanced cardiac output (Rodgers et al., 2009). Also, aged myostatin null mice have heavier hearts compared to the wild-type counterparts and showed physiological cardiac hypertrophy (Jackson et al., 2012).

To understand the function of myostatin in skeletal muscle, novel downstream signaling molecules of myostatin were identified through subtractive hybridization experiments in mice. This test revealed an interesting molecule called Akirin1 (Marshall et al., 2008). Akirin1, a 191 amino acid protein is expressed in a variety of tissues and is likely to be specifically regulated by myostatin in skeletal muscle. Expression of Akirin1 was found to be higher in myostatin null skeletal muscle as compared to wild type muscle (Marshall et al., 2008). Although there is evidence of myostatin-mediated regulation of Akirin1 in skeletal muscle, its role in cardiac muscle is largely undiscovered. Thus, any relevance, if there is, between Akirin1 function in cardiac muscle and onset of cardiac hypertrophy is worth exploring.

**METHODS**

**Mice breeding**

Heterozygote and homozygous breeding pairs of Akirin1 null and wild type mice (C57BL/6 and SV129 background) used in this project were obtained from Dr. Kazufumi Matsushita (Osaka University, Japan). Mice were housed at the Nanyang Technological University (NTU) Animal House, Singapore and maintained on standard chow diet at a constant temperature (20°C) under a 12/12 hour artificial light/dark cycle with unlimited access to water. All experiments were performed as per the approved protocols of Institutional Animal Ethics Committee (IACUC), Singapore.

**Genotype analysis**

Genotype analysis of the Akirin1 transgenic mice was performed according to Ge et al., 2011. A 0.2 x 0.2 cm² section of mouse ear tissue was digested in 300 µl of tissue lysis buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM Na₂EDTA, 0.5% SDS and 100 µg/ml Proteinase K for 2 hour at 56°C. The tissue digest was centrifuged at 13,000 rpm at room temperature for 10 minutes. The supernatant was transferred to a new tube and 500 µl of isopropanol was added followed by 1 ml ice-cold
70% ethanol. The mixture was centrifuged at 13,000 rpm at room temperature for 10 minutes. The supernatant was decanted and DNA pellet was washed with 1 ml of 70% ethanol. After air-drying, the pellet was re-suspended in 100 µl of 10 mM Tris-HCl (pH 8.0) for 5 minutes at 65°C. Semi-quantitative PCR was performed to identify wild type, heterozygous, Akirin1 homozygous knock out alleles using the following oligonucleotides: Akirin1 WT- Forward primer: 5’AGTGATACCAAGGTCTTAGAATCTCC3’; Reverse primer 5’ GTGAAAGTACTGTGACAGCCATCTTG3’; Akirin1 knock-out- Forward primer 5’ ACAAATCCACTCTAAAAGCCATACC3’ Reverse primer 5’ GTTCTATTCCGTCCACATTCACTGTGC3’. PCR reaction conditions were as follows: 5 minutes at 94°C, 35 amplification cycles (30 seconds at 94°C, 1 minute at 67°C, and 1 minute at 72°C) and 10 minutes at 72°C. PCR products were separated on 1% agarose gel in 1X TAE running buffer.

**Muscle sample collection**

Mice were terminated by CO₂ asphyxiation and the hearts were dissected followed by perfusion with 1X PBS and frozen in liquid nitrogen. Body weights and the weights of individual hearts were recorded before sample collection.

**Protein lysate isolation and quantitation**

Cardiac muscle protein lysates were prepared by homogenizing frozen hearts of different genotypes in RIPA buffer (1 ml buffer for 50 mg muscle tissue) using the Yellowline 1KA DI 25 Base Homogenizer (Fisher Scientific, USA) at 9,500-24,000 rpm for 1 minute at each speed keeping on ice. The protein lysate was kept on ice for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected and the protein concentration was measured using Bradford’s protein assay reagent (Bio-Rad, USA) against BSA standard (Bio-Rad, USA) detected at 595 nm.

**SDS-PAGE and western blotting**

Equal amount of proteins, usually 20-30 µg, were mixed with 4 X protein loading dye (Invitrogen, CA, USA) with β-Mercaptoethanol (βMe) (loading dye:βMe = 3:1, V.V). The mixed protein samples were boiled at 100°C for 5 min and separated in SDS-PAGE (NuPage 4-12% gradient Bis-Tris pre-cast polyacrylamide gels, Invitrogen, USA) at 200V (125mA). After electrophoresis, SDS-PAGE gels were transferred to TransBlot (Bio-Rad, USA) nitrocellulose membrane by electroblotting using Xcell II blot module (Invitrogen). The membranes were then stained with Ponceau S (Fluka, Sigma-Aldrich) to determine equal protein loading and transfer efficiency. After de-staining in 1X TBS-T, membranes were blocked overnight with 5% milk in TBS-T at 4°C, or blocked in PVP blocker or BSA blocker for 1 hour at room temperature. Blocking was followed by incubation with specific primary (3 hours for milk and BSA blocker or overnight in PVP blocker) and secondary antibodies (1 hour). Membranes were washed with 1X TBS-T for 5 times (5 minutes each). Primary and secondary antibodies used in this project are listed in supplementary table 2 and 3 respectively. HRP activity was detected using Western Lighting™ Chemiluminescence Reagent Plus (NEL104; PerkinElmer Life Sciences, Waltham, MA, USA). Films (Kodak) were exposed to the membranes and the developed films were analyzed using Quantity One imaging software (Bio-Rad, USA).

**RNA extraction and electrophoresis**

Total RNA from cardiac muscle tissue was isolated using TRizol (Invitrogen, CA, USA) according to the manufacturer’s protocol. 1 ml of TRizol was used for 100 mg cardiac muscle. After homogenization, samples were incubated for 5 minutes at room temperature and centrifuged for 10 minutes at 4°C (12,000 rpm). After mixing with chloroform (200 µl for 1 ml TRizol), samples were vigorously shaken for 15 seconds. Following 3 minutes incubation at room temperature, samples were centrifuged for 15 minutes at 12,000 rpm. The aqueous phase was transferred into fresh tubes and mixed with isopropanol (500 µl for 1 ml TRizol) for RNA precipitation. Total RNA was collected by centrifugation (12,000 rpm) for 10 minutes and the resulting pellet was washed with 75% ethanol and
then re-suspended in DEPC-treated H₂O (0.1% diethylpyrocarbonate). The re-suspended RNA was incubated at 55°C for 10 minutes. After quantification with NanoDrop™ spectrophotometer (ND-1000; NanoDrop Technologies Inc., DE, USA), RNA samples were stored at -80°C. RNA quality was checked by electrophoresis. Equal amount of RNA samples (1 µg), mixed with 2X RNA loading dye, were separated in 1.5% agarose gel (UltraPure™, Invitrogen, USA) in 1X MOPS running buffer with formaldehyde.

cDNA synthesis and quantitative PCR analysis

One µg of RNA was used for cDNA synthesis using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer’s instruction. RNA samples were mixed with 5X iScript reaction buffer and 1 µl of iScript reverse transcriptase (RT). The reaction mixture was incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C and finally for 5 minutes at 85°C. After incubation, synthesized cDNA was stored at -20°C prior to use. Quantitative real time PCR was carried out in triplicates using SsoFast™ EvaGreen supermix and CFX96 Real Time System (Bio-Rad, USA). PCR reaction conditions were as follows: 3 minutes at 98°C and 45 amplification cycles (3 seconds at 98°C, 10 seconds at 60°C, and 10 seconds at 72°C). Gene expression levels were analyzed as fold change using the ∆∆CT (threshold cycle) method, normalized to cyclophilin expression. Details about the oligonucleotides used in this work are listed in supplementary table 1.

Tissue preparation for cryosectioning for histological analysis

Tissue processing was performed according to Ge et al., 2011. For histological analysis, whole heart was embedded with liquid Optimal Cutting Temperature (OCT) compound and then frozen in isopentane cooled with liquid nitrogen. Transverse sections of 10 µm thick were cut using Rotary Cryostat Microtome (RM2265, Leica) and mounted on slides for further histological analysis.

Hematoxylin and Eosin (H & E) staining of muscle sections

Firstly, 10 µm thick sections were cut at the mid-belly region of the whole heart and then Hematoxylin and Eosin staining was performed. The sections were stained in Hematoxylin solution for 1 minute and rinsed in tap water until sections were clear. This was followed by rinsing the sections in Scott’s tap water for 2 minutes and then rinsed with tap water. The heart sections were further stained with Eosin solution for 2 minutes and rinsed with tap water until the sections were clear. The sections were serially dehydrated in 50% ethanol three times, 70% ethanol three times, 90% ethanol for 2 minutes and 100% ethanol for 2 times (2 minutes each). The stained sections were cleared in xylene two times (5 minutes each) and air-dried. The dry sections were mounted using DPX mounting solution and allowed to dry overnight at room temperature. Images were captured using the Leica CTR 6500 microscope, equipped with the Leica DFC 310 FX camera and Image Pro Plus software (Media Cybernetics, Bethesda, MD).

cDNA synthesis and quantitative real time PCR analysis of mature miR-1

One µg of RNA was used for cDNA synthesis using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems®, USA) according to the manufacturer’s instruction. Quantitative real time PCR was carried out in duplicates using Taqman® PCR Kit protocol as per manufacturer’s instruction in CFX96 Real Time System (Bio-Rad, USA). PCR reaction conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C and 40 amplification cycles (15 seconds at 95°C and 1 minute at 60°C). Gene expression levels were analyzed as fold change using the ∆∆CT (threshold cycle) method, normalized to U6 expression. Details about the oligonucleotides used in this project are listed in supplementary table 1.

Statistical analysis

Statistical analysis was performed using one–way ANOVA and two-tailed Student’s t-test. Results
were considered significant at $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***) Results were expressed as mean ± SE of 2 or 3 independent experiments.

RESULTS

1. Identification of Akirin1 knock-out mice by genotyping and confirming the absence of Akirin1 in these mice.

In this study, Akirin1 knock-out mice were used as the animal model to understand the in vivo function of Akirin1. Akirin1 knock-out mice was generated by deleting exon 1 of Akirin1 gene in C57BL6J/SV129 mouse embryos by loxP-cre system. C57BL6J/SV129 (wild type) mice were used as controls in this study (Goto et al., 2008). Mice were bred and genomic DNA was isolated from the ear tissue. To identify the wild type and Akirin1 knock-out pups, semi-quantitative PCR was performed with specific primers. Wild type mice were identified by an amplicon band of 413 base pairs while Akirin1 knock-out mice were identified by an amplicon band of 650 base pairs (Figure 1A). To confirm the absence/reduced Akirin1 mRNA levels in Akirin1 knock-out mice, real time-quantitative PCR was performed on wild type and Akirin1 knock-out quadriceps RNA using Akirin1 specific primers (supplementary Table 1). The Akirin1 Ct values were normalized to that of cyclophilin. Our results clearly indicated that Akirin1 was indeed significantly down regulated in Akirin1 knock-out muscle compared to the wild type muscle (Figure 1B).

Figure 1. Identification of Akirin1 knock-out mice by genotyping and confirming the absence of Akirin1 in these mice. (A) Agarose gel electrophoresis was performed on the PCR amplification products from Akirin1 knock-out and wild type ear explants. 1 kb plus ladder was loaded for reference (Lane 1). Wild type allele was identified by the amplicon band of 413 base pairs (lane 2) while Akirin1 knock-out allele was identified by the amplicon band of 650 base pairs (lane 3). (B) Confirmation of absence of Akirin1 in Akirin1 knock-out muscles. The mRNA expression for Akirin1 was determined in quadriceps muscle obtained from Akirin1 knock-out and wild type mice. The values are mean ± S.E. of four different animals. ***$p<0.001$ denotes significant fold change difference in Akirin1 mRNA expression relative to the wild type.

2. Lack of Akirin1 leads to cardiac hypertrophy.

To understand the effect of Akirin1 in cardiac muscle, we primarily investigated the size of Akirin1 knock-out and wild type hearts. Ex-vivo
Akirin1 knock-out heart showed no significant difference in heart size compared to the wild type (Figure 2A). We examined the weights of the heart and calculated as percentage body weight. The results showed no significant difference in the heart weights between Akirin1 knock-out and wild type mice (Figure 2B). Further, to investigate the effect of Akirin1 on cardiac muscle histology, whole hearts were transversely and longitudinally sectioned and stained with hematoxylin and eosin. The histology images revealed that the ventricular cavities were larger in Akirin1 knock-out heart compared to the wild type heart (Figure 2C).

3. Lack of Akirin1 up-regulates the protein levels of sarcomeric proteins and serum response factor (SRF) in cardiac muscle.

A muscle sarcomere consists of structural and contractile proteins which function jointly leading to the contraction of the muscle. The structural proteins, such as desmin, give the framework and elasticity to the muscle and contractile proteins like actin and myosin, provide force during contraction of the muscle. To investigate if Akirin1 has a novel role in maintaining the levels of these structural and contractile sarcomeric proteins, we wanted to investigate the expression of these proteins in cardiac muscle. For this, Akirin1 knock-out and wild type cardiac muscle protein lysates were subjected to immunoblotting with anti-α-actin, anti-α-MyHC and anti-desmin antibodies. Protein analysis results revealed that Akirin1 knock-out cardiac muscle indeed show significantly increased levels of major sarcomeric proteins like α-actin, α-MyHC and desmin compared to the wild type cardiac muscle (Figure 3A).

Cardiac hypertrophy program has been shown to be induced by various transcription factors expressed in the cardiac muscle like SRF, GATA4 and MEF2C (Hasegawa et al., 1997; Molkentin and Markham, 1993; Zhang et al., 2011). Over-expression of SRF in cardiac muscle is specifically known to activate the cardiac hypertrophy genes like Atrial natriuretic protein (ANP) and β-MyHC via regulating miR-1 expression (Zhang et al., 2001).
Figure 2. Lack of Akirin1 leads to cardiac hypertrophy

(A) A representative image of ex-vivo Akirin1 knock-out and wild type heart. (B) Graph showing the heart weights of Akirin1 knock-out and wild type mice calculated as percentage of total body weight. The values are mean ± S.E. of ten animals. (C) Longitudinal and transverse images of Akirin1 knock-out and wild type hearts stained with hematoxylin and eosin showing larger ventricular cavities in Akirin1 knock-out heart compared to the wild type heart (as represented by *).
Figure 3. Lack of Akirin1 up-regulates the protein levels of sarcomeric proteins and serum response factor (SRF) in cardiac muscle. Western blotting analysis was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates. (A) A representative immunoblot showing the protein levels of various sarcomeric contractile proteins (α-actin, α-MyHC and desmin). GAPDH was used as an internal control for equal protein loading on the gel. B(i) A representative immunoblot showing the protein levels of SRF in Akirin1 knock-out and wild type cardiac muscle. Ponceau S staining was used as an internal loading control on the gel. B(ii) Corresponding normalised densitometry graph of SRF showing significant increase in protein content in Akirin1 cardiac muscle compared to the wild-type (**p<0.01) (n=4). (C) A representative graph showing fold change expression of SRF transcript levels normalised to that of cyclophilin in Akirin1 knock-out cardiac muscle compared to the wild type. The values are mean ± S.E. of four different animals.

As we observed an increase in the levels of sarcomeric proteins in Akirin1 knock-out cardiac muscle, we wanted to investigate if cardiac hypertrophy induced was via SRF in Akirin1 knock-out mice. Protein lysates of Akirin1 knock-out and wild type cardiac muscles were probed with anti-SRF antibody. The western blotting results showed significantly increased SRF protein levels in Akirin1 knock-out cardiac muscle compared to that of the wild type (Figure 3B (i) and 3B (ii)). However, no significant change in the mRNA levels of SRF was observed in Akirin1 knock-out cardiac muscle compared to that of the wild type (Figure 3C). Hence, put together, these results suggest that Akirin1 regulates the expression of SRF post-transcriptionally in cardiac muscle.

4. Akirin1 regulates transcription of miR-1 in cardiac muscle.

Zhang et al. showed that over-expression of SRF in cardiac muscle induced cardiac hypertrophy by down-regulating miR-1 transcription, thereby up-regulating the expression of miR-1 effector genes like ANP and β-MyHC (Zhang et al., 2011). The expression of primary miR-1 levels in both Akirin1 knock-out and wild type cardiac muscle was quantified using real time-qPCR. miR-1 Ct values were normalised to that of U6. Gene expression analysis revealed that Akirin1 knock-out cardiac muscle showed significantly reduced primary miR-1 level compared to wild type cardiac muscle (Figure 4A). As mature miR-1 is the active form that binds to target genes and regulate their expression, the levels of mature miR-1 were analyzed in Akirin1 knock-out and wild type cardiac muscle. Similar to primary miR-1 levels, Akirin1 knock-out cardiac muscle showed significantly lower levels of mature miR-1 when compared to the wild type cardiac muscle (Figure 4B).
Figure 4. Akirin1 regulates transcription of miR-1 in cardiac muscle. Primary and mature miR-1 transcript levels in Akirin1 knock-out and wild type cardiac muscle. mRNA expression analysis was performed on Akirin1 knock-out and wild type cardiac muscle. (A) and (B) Representative graphs showing fold change expression of primary and mature miR-1 transcript levels in Akirin1 knock-out cardiac muscle relative to the wild type respectively. The values are mean ± S.E. of four different animals (**p<0.01).

5. Absence of Akirin1 leads to increased expression of targets of miR-1: Atrial Natriuretic Peptide (ANP) and β-MyHC in cardiac muscle.

As miR-1 levels were down-regulated in Akirin1 knock-out cardiac muscle, we investigated the expression levels of some of the target genes regulated by miR-1 like Atrial natriuretic protein (ANP) and β-MyHC. Immunoblotting was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates with anti-ANP and anti-β-MyHC antibodies. The protein expression results showed that Akirin1 knock-out cardiac muscle expressed significantly increased levels of both ANP and β-MyHC protein compared to the wild type cardiac muscle [Figure 5A(i), B(i),A(ii) and S5B(ii)]. To determine if the increase in ANP and β-MyHC protein levels is attributed to increased ANP and β-MyHC mRNA levels, gene expression analysis was performed to quantify their mRNA levels by real time-qPCR with cyclophilin as normalising internal control. The results indicated that Akirin1 knock-out cardiac muscle showed significantly higher levels of both ANP and β-MyHC mRNA compared to the wild type cardiac muscle (Figure 5C and SD).

6. Absence of Akirin1 in the cardiac muscle leads to activation of IGF-1/Akt/mTOR pathway leading to cardiac hypertrophy.

To investigate the mechanism behind cardiac hypertrophy seen in Akirin1 knock-out cardiac muscle, we investigated certain important cardiac hypertrophy inducing signaling pathways like IGF-1/Akt/mTOR pathway. This pathway involves a cascade of intracellular components which together signal increased protein synthesis thus inducing cardiac hypertrophy (Haq et al., 2000; McMullen et al., 2003). Firstly, we investigated the protein level of the IGF-1 growth factor. Protein lysates from Akirin1 knock-out and wild type cardiac muscle were subjected to western blotting with anti-IGF-1 antibody. Western blotting results showed that Akirin1 knock-out cardiac muscle have significantly increased levels of intracellular IGF-1 levels compared to the wild type cardiac muscle [Figure 6.1A(i)]. Densitometry analysis proved that the IGF-1 levels were significantly up-regulated in Akirin1 knock-out cardiac muscle compared to the wild type [Figure 6.1A(ii)]. Binding of IGF-1 to its receptor activates its downstream target, phosphatidyl inositol-3-Kinase (PI3-K). PI3-K helps in phosphorylating membrane phospholipid phosphoinositide-4,5-biphosphate (PIP2) to phosphoinositide-3,4,5-triphosphate (PIP3). So we looked at the levels of both active phosphorylated PI3-K p85 subunit levels and total p33-K p85 subunit levels using western blotting. Akirin1 knock-out and wild type cardiac muscle protein lysates were probed with anti-phosho-PI3-K p85 (tyr458)/p55 (tyr199) and anti-PI3-K p85 antibodies. Protein expression analysis showed significantly increased levels of phosho-PI3-K p85 (tyr458)/p55 (tyr199) level in Akirin1 knock-out compared to the wild type cardiac muscle. However, the total PI3-K p85 level remained unchanged [Figure 6.1B(i)]. Densitometry analysis showed that Akirin1 knock-out cardiac muscle have significantly higher level of phospho-PI3-K p85 (tyr458)/p55 (tyr199) compared to the wild type cardiac muscle while the total PI3-K p85 level remained unchanged [Figure 6.1B(ii) and B(iii)]. The ratio of phospho-PI3-K p85 (tyr458)/p55 (tyr199) to total PI3-K p85 was significantly higher in Akirin1 knock-out cardiac muscle compared to that of the wild type (Figure6.1B(iv)).

The signaling molecule that act downstream of PI3-K is Akt. Akt also called protein kinase B is a serine/threonine protein kinase and is activated when phosphorylated at ser 473. We investigated...
the levels of both phospho-Akt (ser473) and total-Akt in Akirin1 knock-out and wild type cardiac muscle. The immunoblotting results showed a significant increase in phosphorylation of Akt protein in Akirin1 knock-out cardiac muscle compared to the wild type cardiac muscle, while the total Akt levels remained unchanged [Figure 6.2A(i)]. Densitometry analysis showed significant increase in Akt phosphorylation in Akirin1 knock-out cardiac muscle compared to the wild type cardiac muscle with no changes in the total Akt levels [Figure 6.2A(ii) and A(iii)]. The ratio of phospho-Akt (ser473) to total Akt was higher in Akirin1 knock-out cardiac muscle compared to the wild type cardiac muscle [Figure 6.2A(iv)]. Akt can further stimulate protein synthesis pathway via mammalian target of rapamycin (mTOR) and Glycogen synthase kinase-3-beta (GSK3β). Akt promotes protein synthesis by phosphorylating mTOR at ser2448 while inactivates GSK3b by phosphorylating at ser9 (Manning and Cantley, 2007). Hence, we estimated the phosphorylated and total levels of both mTOR and GSK3β in Akirin1 knock-out and wild-type cardiac muscle. Firstly, western blotting was performed on Akirin1 knock-out and wild type cardiac muscle with anti-phospho-mTOR (ser2448) and anti-mTOR antibodies. Protein expression analysis showed that Akirin1 knock-out cardiac muscle have significantly increased levels of both phospho-mTOR (ser2448) and total mTOR compared to the wild type cardiac muscle (Figure 6.2B(i), B(ii) and B(iii)]. The ratio of phospho-mTOR (ser2448) to total mTOR was also higher in Akirin1 knock-out cardiac muscle compared to the wild type [Figure 6.2B(iv)].

Further, western blotting was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates with anti-phospho-GSK3β (ser9) and anti-GSK3β antibodies. Akirin1 knock-out cardiac muscle showed increased protein levels of both phospho-GSK3β (ser9) and total GSK3β compared to the wild type cardiac muscle (Figure 6.3A(i), A(ii) and A(iii)). The ratio of phospho-GSK3β (ser9) to total GSK3β was also significantly up-regulated in Akirin1 knock-out cardiac muscle compared to the wild type cardiac muscle [Figure 6.3A(iv)]. mTOR activates translation of various cellular proteins by activating eukaryotic translation initiation factor 4E (eIF4E) by phosphorylating the inhibitory eIF4E-binding proteins called 4eBPs. Western blotting was performed on protein lysates from Akirin1 knock-out and wild type cardiac muscle with anti-phospho-4eBP1 (ser65/thr70) and anti-4eBP1 antibodies. The levels of both phospho-4eBP1 (ser65/thr70) and total 4eBP1was up-regulated in Akirin1 knock-out cardiac muscle compared to the wild type [Figure 6.3B(i), B(ii) and B(iii)]. The ratio of phospho-4eBP1 (ser65/thr70) to total 4eBP1 was also significantly increased in Akirin1 knock-out cardiac muscle compared to the wild type cardiac muscle [Figure 6.3B(iv)]. All these observations together suggest that in the absence of Akirin1, the signaling proteins involved in IGF-1/Akt/mTOR pathway are activated leading to cardiac hypertrophy phenotype.
Figure 5. Absence of Akirin1 leads to increased expression of targets of miR-1: Atrial Natriuretic Peptide (ANP) and β-MyHC in cardiac muscle. Expression analysis of downstream targets of miR-1 namely ANP and β-MyHC in Akirin1 knock-out and wild type cardiac muscle. Western blotting analysis was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates. A(i) and B(i) Representative immunoblots showing the protein levels of ANP and β-MyHC respectively. GAPDH was used as an internal control for equal protein loading on the gel. A(ii) and B(ii) Corresponding densitometry graphs of ANP and β-MyHC showing significant increase in protein content in Akirin1 cardiac muscle compared to the wild type (**p<0.01) (n=4). mRNA expression of ANP and β-MyHC was determined in Akirin1 knock-out and wild type cardiac muscle. (C) and (D) Representative graphs showing the fold change mRNA expression of ANP and β-MyHC in cardiac muscle respectively. The values are mean ± S.E. of four different animals (**p<0.01).
Figure 6.1. Absence of Akirin1 in the cardiac muscle leads to increased levels of IGF-1 and PI3-K proteins. Western blotting analysis was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates. A(i) A representative immunoblot showing the protein levels of IGF-1 in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an internal control. A(ii) Densitometry graph of IGF-1 protein levels in Akirin1 knock-out and wild type cardiac muscle. B(i) A representative immunoblot showing the protein levels of both phospho-PI3-K p85 (tyr 458)/p55(tyr 199) and total PI3-K p85 in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an internal control. B(ii) and B(iii) Densitometry graphs of phospho-PI3-K p85 (tyr 458)/p55(tyr 199) and total PI3-K p85 protein levels in Akirin1 knock-out and wild type cardiac muscle.
type cardiac muscle respectively. B(iv) Densitometry graph representing the ratio of phospho-P13-K p85 (tyr 458)/p55(tyr 199) to total P13-K p85 levels. All the graph values are mean ±S.E. of four different animals. Level of significance was compared to wild type (*p<0.05 and **p<0.01).

Figure 6.2. Absence of Akirin1 in the cardiac muscle leads to increased levels of Akt and mTOR proteins. Western blotting analysis was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates. A(i) A representative immunoblot showing the protein levels of both phospho-Akt (ser 473) and total Akt in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an
internal control. A(ii) and C(iii) Densitometry graphs of phospho-Akt (ser 473) and total Akt protein levels in Akirin1 knock-out and wild type cardiac muscle respectively. A(iv) Densitometry graph representing the ratio of phospho-Akt (ser 473) to total Akt levels. B(i) A representative immunoblot showing the protein levels of both phospho-mTOR (ser 2448) and total mTOR in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an internal loading control. B(ii) and B(iii) Densitometry graphs of phospho-mTOR (ser 2448) and total mTOR protein levels in Akirin1 knock-out and wild type cardiac muscle respectively. B(iv) Densitometry graph representing the ratio of phospho-mTOR (ser 2448) to total mTOR levels. All the graph values are mean ±S.E. of four different animals. Level of significance was compared to wild type (*p<0.05 and **p<0.01).
Figure 6.3. Absence of Akirin1 in the cardiac muscle leads to increased levels of GSK3β and 4eBP1 proteins.

Western blotting analysis was performed on Akirin1 knock-out and wild type cardiac muscle protein lysates. A(i) A representative immunoblot showing the protein levels of both phospho-GSK3β (ser 9) and total GSK3β in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an internal loading control. A(ii) and A(iii) are densitometry graphs of phospho-GSK3β (ser 9) and total GSK3β protein levels in Akirin1 knock-out and wild type cardiac muscle respectively. A(iv) Densitometry graph representing the ratio of phospho-GSK3β (ser 9) and total GSK3β levels. B(i) A representative immunoblot showing the protein levels of both phospho-4eBP1 (ser 65/thr 70) and total 4eBP1 in Akirin1 knock-out and wild type cardiac muscle. GAPDH was used as an internal control for equal protein loading. B(ii) and B(iii) Densitometry graphs of phospho-4eBP1 (ser 65/thr 70) and total 4eBP1 protein levels in Akirin1 knock-out and wild type cardiac muscle respectively. B(iv) Densitometry graph representing the ratio of phospho-4eBP1 (ser 65/thr 70) to total 4eBP1 levels. All the graph values are mean ±S.E. of four different animals. Level of significance was compared to wild type (*p<0.05, **p<0.01 and ***p<0.001).

DISCUSSION

Akirins have been very recently identified as conserved nuclear factors with the presence of nuclear localization signal at the N-termini justifying their cellular localization (Goto et al., 2008). Recent work has specifically revealed the diverging functions between mouse Akirin1 and its isoform Akirin2 as obvious from the conflicting phenotypes of the respective Akirin1 and Akirin2 knock-out mice. Whereas the mouse Akirin 2 has been shown to be playing pivotal role during immune and inflammatory responses in drosophila as well as in mice, owing to its obligate function downstream of TLR, TNF and IL-1β signaling at the level of the transcription factor NF-kB for the release of IL-6 (Goto et al., 2008), the Akirin1 knock-out mice showed no obvious phenotype. Hence, the precise function of Akirin1 still remains obscure.

Akirin1 is a recently discovered pro-myogenic molecule that has not been extensively studied. Being a ubiquitously expressed nuclear protein, it can be speculated to have different functions in different tissues. Some of the important discoveries in the past revealed its role in myogenic differentiation being a downstream target of myostatin and negatively regulated by it (Marshall et al., 2008). Also, Akirin1 has been shown to be involved in satellite cell activation and skeletal muscle regeneration (Salerno et al., 2009). However, the functional role of Akirin1 in the other striated muscle i.e. cardiac muscle has been less well defined till date. Therefore, to further elucidate precisely the function of Akirin1 in cardiac muscle, Akirin1 knock-out mouse model was used in this study. The results obtained in this study show that Akirin1 knock-out mice did not show any obvious phenotypic change when compared to wild-type mice. However, Akirin1 was found to display a distinctive function in cardiac muscle. Our results showed that Akirin1 knock-out heart had larger ventricular cavity as seen in left ventricular hypertrophy (LVH) (Figure 2). Larger ventricular cavities have been one of the clinical manifestations of cardiac hypertrophy as seen in athletes with hypertrophic cardiomyopathy (Sheikh et al., 2015). Previous studies have highlighted increased levels of Insulin-like growth factor 1 (IGF-1) in professional athletes than in control subjects (Neri Serneri et al., 2001) and supportively serum levels of IGF-1 was elevated in humans after exposure to exercise or physical training (Koziris et al., 1999; Yeh et al., 1994). The IGF-1 acts via its receptor (IGFIR), activates PI3-K (p110a; class IA) which then activates Akt/mTOR pathway to increase translation of downstream targets. Thus, additionally the PI3-K/Akt/GSK3β pathway is implicated in both pathological and physiological hypertrophy and an initial activation of PI3-K leads therefore to cardiac hypertrophy (Naga Prasad et al., 2000). However, more recently, substantial studies have proved the essentiality of Akt1 for physiological rather than pathological heart
growth. Also, the transgenic mice exhibiting enhanced cardiac IGF1/PI3-K (p110α) signaling developed cardiac hypertrophy, although life span was normal and cardiac function was either normal or enhanced (McMullen et al., 2003; Reiss et al., 1996; Shioi et al., 2000). Consistent to all these findings, increase in IGF-1 and PI3-K proteins was seen in Akirin1 knock-out cardiac muscle leading to activation of PI3-K/Akt/GSK3β pathway resulting in augmented protein synthesis (Figure 6). This increased protein synthesis was proven by increased protein levels of sarcomeric and contractile proteins like α-actin, MyHC and desmin in our study (Figure 2). Apart from PI3-K/Akt/GSK3β pathway, various other stress kinase pathways are known to be activated during pathological cardiac hypertrophy like ERK pathway, p38 MAPK pathway and JNK pathway (Bueno et al., 2000; Bueno and Molckentin, 2002; Ramirez et al., 1997; Sugden and Clerk, 1998; Wang et al., 1998a; Wang et al., 1998b; Zhang et al., 2003). The results from our study also showed that all the above pathways were activated in the absence of Akirin1 (Figure S1, S2 and S3). Thus our study clearly show that ablation of Akirin1 affects the cardiac muscle mass homeostasis and might be critical for induction of cardiac hypertrophy. Together, the data from our work and previous studies are indicative of possibility that targeting genes like Akirin1 might prove advantageous in treatment of cardiomyopathy.

Zhang et al. showed that cardiac specific over-expression of SRF led to cardiomyopathy (Zhang et al., 2001). Later, the same research group in 2011 showed that cardiac-specific over-expression of SRF led to down-regulation of miR-1 biogenesis (Zhang et al., 2011). Evidently, it was also shown that cardiac muscle specific miR-1 was a direct target of SRF and its potent co-activator myocardin and are involved in negative regulation of ventricular cardiomyocyte proliferation (Zhao et al., 2005). Similarly, another study also supported the direct role of SRF and miR-1 in failing heart. The study showed that reduced levels of SRF also down-regulates miR-1 transcription affecting the downstream target proteins i.e. sodium/calcium exchanger NCX1 and Annexin A5 during heart failure (Tritsch et al., 2013). In another study it was shown that miR-1 targets cardiac hypertrophy inducing genes like ANP and β-MyHC (Wei et al., 2014). Consistent with these findings, our study also showed that in the absence of Akirin1, SRF protein level was up regulated in the cardiac muscle (Figure 3). This increase in SRF might be leading to down regulation of miR-1 biogenesis due to which ANP and β-MyHC expression was up regulated (Figure 4 and 5). Re-expression of fetal genes like ANP and β-MyHC are proven to induce Akt signaling pathway in the cardiac muscle leading to increased sarcomeric protein synthesis and pathological hypertrophy (Kato et al., 2005). Thus, we speculate that Akirin1 post-transcriptionally regulates SRF levels in cardiac muscle leading to cardiac hypertrophy through miR-1. Although the involvement of Akirin 1 in the specific type of cardiac hypertrophy (pathological or physiological) needs further investigation. Furthermore, the probable function of Akirin1 in skeletal myogenesis cannot be sidelined and might prove to be equally important and is beyond the scope of this study.

Figure 7. A schematic diagram summarizing the various results obtained in understanding the
The possible role of Akirin1 in inducing cardiac hypertrophy.

Based on our results, we speculate that loss of Akirin1 in cardiac muscle results in the post-transcriptional up regulation of the SRF in the cardiac muscle due to unknown/unidentified mechanism or intermediary factors resulting in the attenuation of biogenesis of miR-1. Subsequently, an increased expression is observed in the decisive genes responsible for the induction of cardiac hypertrophy, ANP and β-MyHC (as hypothesized in schematic diagram shown in Figure 7). In parallel, it has been well established that IGF-1 and IGF-1 receptor are targets of miR-1 and there exists an inverse correlation between miR-1 and IGF-1 in models of cardiac hypertrophy and failure as well as in the C2C12 skeletal muscle cell model of differentiation (Elia et al., 2009). Hence, down regulation of miR-1 leads to increased levels of IGF-1, further activating the PI3-K/Akt/mTOR pathway resulting in increased translation of sarcomeric proteins like α-actin, MyHC and desmin within target (cardiac muscle) thereby leading to induction of cardiac muscle hypertrophy (Figure 7).

In summary, we have observed that Akirin1 regulates certain genes at transcription level and others post-transcriptionally. The exact mode of Akirin1 action is yet to be elucidated and it is quite possible that many of the observed differences are due to redundant or indirect processes to which Akirin1 is the contributing factor. For example, Akirin1 may be regulating its target genes post-transcriptionally either by affecting the mRNA stability or through micro-RNAs inhibiting translation of target genes. Hence, we speculate that Akirin1 possibly regulates cardiac hypertrophy through novel unexplored mechanisms. However, it is inconclusive to attribute a precise function for Akirin1 in pathological or physiological hypertrophy at this point. Nonetheless, cardiovascular research and therapy always has been in the pursuit of novel molecules responsible for onset of pathological processes and our study opens up new vistas to target one such contributing factor in cardiac hypertrophy. Further, identifying distinctive mechanisms and exclusive factors responsible for physiological heart growth and induction of cardiac hypertrophy may pave the way for development of improved pharmacotherapies for protecting and maintaining the failing heart.

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Conflict of interest statement

The author declares no competing or conflict of interests. The funders had no role in study design, writing of the manuscript and decision to publish.

Authors’ contributions

Vanitha V. Rao conceptualized and performed all the experiments in this manuscript. S. Umamaheswari and K. ArockiaMary provided technical help in some of the western blotting and qPCR experiments. Kazufumi Matsushita and Shizuo Akira are duly acknowledged for providing the mouse model used in the study along with the genotyping analysis. Vanitha V. Rao and Abhishek Mohanty co-wrote this manuscript.

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